Integrated Transcriptome Sequencing (RNA-seq) and Proteomic Studies Reveal Resource Reallocation towards Energy Metabolism and Defense in *Skeletonema marinoi* in Response to CO₂ Increase

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**ABSTRACT** Rising atmospheric CO₂ concentrations are causing ocean acidification (OA) with significant consequences for marine organisms. Because CO₂ is essential for photosynthesis, the effect of elevated CO₂ on phytoplankton is more complex, and the mechanism is poorly understood. Here, we applied transcriptome sequencing (RNA-seq) and iTRAQ proteomics to investigate the impacts of CO₂ increase (from ca. 400 to 1,000 ppm) on the temperate coastal marine diatom *Skeletonema marinoi*. We identified 32,389 differentially expressed genes and 1,826 differentially expressed proteins from conditions where the CO₂ is elevated, accounting for 48.5% of the total genes and 25.9% of the total proteins we detected, respectively. Elevated partial CO₂ pressure (pCO₂) significantly inhibited the growth of *S. marinoi*, and the “omic” data suggested that this might be due to compromised photosynthesis in the chloroplast and raised mitochondrial energy metabolism. Furthermore, many genes/proteins associated with nitrogen metabolism, transcriptional regulation, and translational regulation were markedly upregulated, suggesting enhanced protein synthesis. In addition, *S. marinoi* exhibited higher capacity of reactive oxygen species production and resistance to oxidative stress. Overall, elevated pCO₂ seems to repress photosynthesis and growth of *S. marinoi* and, through massive gene expression reconfiguration, induce cells to increase investment in protein synthesis, energy metabolism, and antioxidative stress defense, likely to maintain pH homeostasis and population survival. This survival strategy may deprive this usually dominant diatom in temperate coastal waters of its competitive advantages in acidified environments.

**IMPORTANCE** Rising atmospheric CO₂ concentrations are causing ocean acidification with significant consequences for marine organisms. Chain-forming centric diatoms of *Skeletonema* is one of the most successful groups of eukaryotic primary producers with widespread geographic distribution. Among the recognized 28 species, *Skeletonema marinoi* can be a useful model for investigating the ecological, genetic, physiological, and biochemical characteristics of diatoms in temperate coastal regions. In this study, we found that the elevated pCO₂ level seems to repress photosynthesis and growth of *S. marinoi* and, through massive gene expression reconfiguration, induce cells to increase investment in protein synthesis, energy metabolism, and antioxidative stress defense, likely to maintain pH homeostasis and population survival. This survival strategy may deprive this usually dominant diatom in temperate coastal waters of its competitive advantages in acidified environments.

**KEYWORDS** metabolism, ocean acidification, proteomics, RNA-seq, *Skeletonema marinoi*


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Since industrialization, anthropogenic CO₂ has been quickly accumulating in the atmosphere, resulting in global warming and ocean acidification. It is estimated that atmospheric CO₂ will rise from the current level of 400 to 1,000 ppm by the end of this century (1). As the surface seawater absorbs additional atmospheric CO₂, perturbing ocean carbonate buffering system, the consequent decreased pH can have profound effects on primary producers, as well as other organisms in the ocean (2). However, it is still a controversial issue how ocean acidification will change marine phytoplankton community structure and productivity because the combined effect of elevated CO₂ and decreased pH can vary between species. Diatoms are a major group of primary producers in the ocean, which contribute ~40% of the annual marine primary productivity (3) and exhibit higher carbon sequestration into the deep sea because their silica frustules improve their sinking rates (4). The effects of ocean acidification on diatoms can have significant biogeochemical impacts. Diatoms’ responses to rising partial CO₂ pressure (pCO₂) and dropping pH are species specific, with potential winners, neutrals, and losers among the species (5–8). For instance, elevated pCO₂ had insignificant effects on the growth, photosynthesis, dark respiration, particulate organic carbon, and particulate organic nitrogen of diatom *Skeletonema pseudocostatum* (9). Under higher pCO₂, CO₂-driven acidification decreased the productivity of *Thalassiosira pseudonana* by increasing dark respiration (10). In contrast, rising temperature and CO₂ (25°C, 1,000 ppm) caused increases in the growth rate, pigment composition, and biochemical productivity of *Skeletonema dohrnii* (11).

The effects of elevated CO₂ on phytoplankton are more complex than on animals, because higher CO₂ without considering acidification are favorable to photosynthesis. Normally, diatom species rely on CO₂-concentrating mechanisms (CCMs) to obtain sufficient CO₂ for Rubisco, the enzyme that fixes CO₂ (12, 13). Generally, diatoms downregulate their CCMs under enhanced pCO₂ (14), potentially saving energy required by CCMs. Decreased pH, in contrast, could disturb cell surface and intracellular pH stability, so that diatom cells (as well as animals) require additional energy to maintain pH homeostasis (5, 15). Moreover, rising CO₂ can have broader metabolic effects, leading to changes in biochemical composition of diatom cells, for instance, the levels of pigments, fatty acids, lipids, amino acids, proteins, nucleic acids, particulate organic carbon, and particulate organic nitrogen (9, 11, 16). These changes will occur through transcriptional and translational regulation.

Chain-forming centric diatoms of *Skeletonema* is one of the most successful groups of eukaryotic primary producers with widespread geographic distribution (9, 17, 18). Among the recognized 28 species, *S. marinoi* can be a useful model for investigating the ecological, genetic, physiological, and biochemical characteristics of diatoms in temperate coastal regions (19). It is not only an important primary producer for the higher trophic levels but also contributes significantly to harmful algal blooms in spring (17). In addition, *S. marinoi* plays an important role in the sequestration and mineralization of carbon, silicon, phosphorus, and nitrogen in the marine biogeochemical cycle (20). To gain understanding on how this species will respond to increasing CO₂, we performed transcriptome and proteome analyses on *S. marinoi* growing under an elevated CO₂ condition. Our results provide insights into potential ecological consequences to this and other related diatoms of increasing CO₂ in future oceans.

**RESULTS**

*Skeletonema marinoi* growth and pH trends. During the 5-day experimental period, the similar growth trends were observed for ambient CO₂ and elevated CO₂ treatment groups with maximum cell density at (4.64 ± 0.03) × 10⁶ and (4.05 ± 0.07) × 10⁶ cells ml⁻¹ (one-way analysis of variance [ANOVA], *P* < 0.01), respectively (Fig. 1A). Under bubbling with ambient CO₂, *S. marinoi* achieved an intrinsic growth rate (μ) of 0.96 ± 0.002 day⁻¹, while bubbling with elevated CO₂ significantly inhibited the cell growth and decreased growth rate to 0.92 ± 0.004 day⁻¹ (one-way ANOVA, *P* < 0.01). The highest growth rate in both groups occurred on the first day, at 1.57 ± 0.02 day⁻¹ (ambient pCO₂) and 1.52 ± 0.08 day⁻¹ (elevated pCO₂), respectively (one-way ANOVA, *P*
> 0.05). We noticed that the pH fluctuations in the treatment of ambient CO₂ and elevated CO₂ were different. As the cell density increased, pH values under ambient CO₂ gradually rose from day 1 to day 5, while the pH under elevated CO₂ showed a slight decrease during this period (Fig. 1B).

**Transcriptome assembly, annotation, and protein identification.** After quality filtering for raw reads, more than 44.5 M clean transcript reads were obtained from each sample. Approximately 84% of clean transcript reads found matches in the reference databases. On average, 38.5 and 46.5% uniquely mapping reads were generated in the ambient CO₂ group and elevated CO₂ group, respectively (see Table S1 in the supplemental material). In the transcriptome analysis, we achieved 66,873 unigenes with 25,441 upregulated genes and 6,948 downregulated genes. Simultaneously, iTRAQ-based quantitative proteomics yielded 32,965 peptides and 7,055 proteins with 1,246 upregulated proteins and 580 downregulated proteins. In total, 6,831 gene transcripts were correlated with their corresponding proteins. Among them, 1,107 differentially expressed proteins (DEPs; upregulated, \( n = 938 \); downregulated, \( n = 169 \)) exhibited the same differential expression trends between normal and elevated CO₂ conditions (Table 1 and Fig. 2).

The number of differentially expressed genes (DEGs) and DEPs assigned to Gene Ontology (GO) terms were 13,739 and 1,326, which accounted for 20.6% of total detected genes and 18.8% of total detected proteins, respectively. The DEGs were defined and described by GO functional enrichment analysis, and 25 significantly enriched GO terms (Q value < 0.05) were identified in our transcriptome sequencing (RNA-seq) data. For instance, calcium ion binding, potassium ion transport, metal ion binding, mitogen-activated protein (MAP) kinase activity, Arp2/3 protein complex, and phosphorus-oxygen lyase activity were strongly regulated at the mRNA level (Fig. 3A). According to our iTRAQ data, 207 enriched GO terms (\( P < 0.05 \)) were identified, showing that the elevated CO₂ group exhibited enrichment of ribosome, translation, cellular nitrogen compound metabolic process, protein metabolic process, gene expression, nucleic acid binding, among others (Fig. 3B; see Table S2). Interestingly, only three significantly enriched GO terms were found in both our RNA-seq and iTRAQ data, including intracellular, motile cilium, and...
and MAP kinase activity (Fig. 3A and B; see Table S2).

To determine the overall functional distribution of DEGs/DEPs in the two CO2 treatment groups, reads were subjected to KEGG Orthology (KO) identifiers, and a total of 9,057 DEGs and 788 DEPs were identified. We also performed a functional enrichment analysis for DEGs and DEPs through the KEGG pathway, finding that only RNA transport was enriched in both RNA-seq and iTRAQ data. Besides, 13 pathways (Q value < 0.05) were also enriched by annotated DEGs, including proteasome, nucleotide excision repair, sphingolipid metabolism, tryptophan metabolism, galactose metabolism, carbon metabolism, etc. (Fig. 4). A total of 11 significantly enriched KEGG pathways (P < 0.05) were identified in our iTRAQ data, and these pathways were ribosome, citrate cycle (tricarboxylic acid [TCA] cycle), aminoacyl-tRNA biosynthesis, oxidative phosphorylation, phagosome, N-glycan biosynthesis, and fatty acid metabolism, among others (Fig. 4).

Nitrogen metabolism and transporter of nutrients. In total, 48 nitrogen metabolism associated genes were identified from our transcriptomic data, among which 30 showed differential expression. Most of DEGs were upregulated, including genes involved in N transport and assimilation, with the exception of a few genes involved in amino acid transport, urea cycle (OUC), and glutamine synthetase/glutamate synthase (GS-GOGAT) that were downregulated under elevated pCO2 (Fig. 5; see also Table S3 in the supplemental material). Form our proteomic data, 36 proteins were associated with nitrogen metabolism, and 11 of them showed strong regulation. Except for one ammonium transporter (AMT), the remaining 10 DEPs, which functioned in N transport, OUC, and GS-GOGAT, were all upregulated under elevated pCO2 (Fig. 5; see also Table S3).

In addition to N-related transport genes, we also identified other 65 transport-related genes from our transcriptomic data, among which 47 were differentially
FIG 3: Top 30 ranking GO terms found in the transcriptome in the proteomic analysis. (A) Top 30 ranking GO terms found in the transcriptome. Red text indicates significantly enriched GO terms (Q value < 0.05), and the color strength represents the Q value. (B) Top 30 ranking GO terms found in the proteome. Red text indicates significantly enriched GO terms (P < 0.05), and the color strength represents the P value.
expressed, including 27 upregulated and 20 downregulated (Fig. 6; see also Table S3). For example, two inorganic phosphate transporter (PiT), Ca\(^{2+}\)-activated K\(^+\) channel slowpoke alpha subunit (KV,Ca), metal transporter CNMM (CNMM), potassium channel (K channel), and ABC transporter F family were upregulated under the elevated CO\(_2\) condition. In contrast, one PiT, copper transporter (COT), sulfate permease (SUP), sulfate transporter (SUT), zinc transporter (ZiT), and ABC transporter A/C/D/G families were downregulated under the elevated CO\(_2\) condition. In our proteomic data set, we detected 36 proteins associated with nutrient transport (except nitrogen), among which 6 were upregulated and 2 were downregulated (Fig. 6; see also Table S3). The abundance of KV,Ca, PiT, CNMM, K channel, and ABC transporter B/F family proteins significantly increased under the elevated CO\(_2\) condition, while the abundance of ABC transporter A/G family proteins significantly decreased under the elevated CO\(_2\) condition.
Photosynthesis, electron transport chain, and carbon fixation. In search of DEGs/DEPs involved in photosynthesis, we found that 19 light-harvesting complex II chlorophyll a/b binding (Lhca) genes (2 upregulated, 17 downregulated) and 3 Lhca proteins (downregulated) showed strong regulation under the elevated CO2 condition (Fig. 6; see Table S4). All the photosystem II reaction center (PSII RC) genes (PsbA, PsbB, PsbC, and PsbE) identified in our RNA-seq data were upregulated, but this was not reflected in our iTRAQ data (Fig. 6; see Table S4). However, both transcriptome and proteome data indicated that the oxygen evolving complex (OEC) was downregulated under the elevated CO2 condition (Fig. 6; see Table S4). This would strongly suggest accumulation of unassembled PSII repair cycle intermediates, because it is not possible to assemble a complete PSII without the oxygen-evolving complex. Form the core of the photosystem I (PSI) complex, PsA genes were upregulated transcriptionally but not so from the proteomic data set (Fig. 3; see also Table S4).

In search of DEGs associated with photosynthesis electron transport, we observed...
FIG 6 Heatmap showing changes in expression levels of genes or proteins involved in the transport of nutrients, calcium signaling, ROS homeostasis, cell death, photosynthesis, and carbon fixation. The color scale represents normalized gene or protein expression values (log2-fold change) on a column z-score, increasing from blue (lowest) to red (highest). NA, not available.
significantly increased expression of PSI RC, PSII RC, and ATP synthase (chloroplast) and decreased expression of cytochrome b6-f complex iron-sulfur subunit (petC), cytochrome c6 (CytC6), ferredoxin (Fd), and ferredoxin-NADP+ reductase (FNR) (Fig. 6; see Table S4). All DEPs involved in photosynthesis electron transport showed significant downregulation, including Fd, CytC6, and ATP synthase (chloroplast) proteins (Fig. 6; see Table S4). In addition, genes/proteins associated with C3 and C4 photosynthesis were also identified in our study, and some of them were statistically significant. Notably, proteins encoding ribulose 1,5-bisphosphate carboxylase large chain (RBCL), carbonic anhydrase (CA), phosphoenolpyruvate carboxylase 1 (PEPC1), phosphoenolpyruvate carboxykinase (PEPCK) all displayed weak downregulation (not significant) (Fig. 6; see Table S4).

In both our RNA-seq and proteomic data sets, many genes/proteins involved in mitochondrial electron transport chain were upregulated (Fig. 7A; see Table S5), indicating that the electron transfer in the mitochondria was enhanced under the elevated CO2 condition. Notably, complex V (ATP synthase) couples the transfers of electron back into the matrix to produce ATP by oxidative phosphorylation (OXPHOS). We observed that OA-driven upregulation of the ATP synthase associated with OXPHOS, including H+-transporting ATPase, F-type H+-transporting ATPase, V-type H+-transporting ATPase (Fig. 7A; see Table S5). These results suggested that ATP synthesis in mitochondria was promoted under the elevated CO2 condition.

RNA and protein syntheses and processing. RNA-seq analysis indicated that 15 genes encoding RNA polymerase I (Pol I), 10 genes associated with Pol II, and 17 genes related to Pol III were differentially expressed under the elevated CO2 condition. except for one RPABC5 gene, all the DEGs were all significantly upregulated under the elevated CO2 condition (Fig. 7B; see Table S6). This upregulation trend was reflected in the proteomic data set, specifically for RPB1, RPB3, RPAC1, and RPAC2 proteins (Fig. 7B; see Table S6). These results suggested that RNA synthesis was enhanced under the elevated CO2 condition.

In this study, 31 genes [e.g., 5′-3′ exoribonuclease, poly(A)-specific RNase mRNA-decapping enzyme, mRNA-decapping enzyme 1B] associated with RNA degradation were found to significantly change expression under the elevated CO2 condition. Most of them were upregulated, indicating upregulation of the cellular RNA degradation (Fig. 7B; see Table S6). Differential expression analysis indicated that seven DEPs involved in RNA degradation. Of these, three DEPs were upregulated, while other four DEPs were down-regulated under the elevated CO2 condition (Fig. 7B; see Table S6).

In the protein synthesis machinery, we identified 62 genes associated with eukaryotic translation initiation factors (eIFs). Among them, 43 eIF genes were found to significantly change expression under the elevated CO2 condition, and all these DEGs were upregulated (Fig. 7B; see Table S6). Besides, our iTRAQ data revealed a set of 23 DEPs associated with eIFs, 20 of which were remarkably upregulated, such as translation initiation factor 1 (eIF1) and two translation initiation factor 4A (eIF4A) (Fig. 7B; see Table S6). In addition, from our transcriptomic data set, we found 51 DEGs (44 upregulated, 7 downregulated) associated with protein processing in the endoplasmic reticulum (ER) (Fig. 7B; see Table S6). From our proteomic data set, we found 23 ER-related proteins that were regulated under the elevated CO2 condition, and All of these DEPs were upregulated (Fig. 7B; see Table S6). These results suggested the protein synthesis and processing machinery may be promoted under the elevated CO2 condition.

In addition, we found 79 genes involved in ER-associated protein degradation, of which 37 were upregulated, while 13 were found to downregulated under the elevated CO2 condition (Fig. 7B; see Table S6). Meanwhile, our proteomic analysis indicated 53 proteins related to ER-associated protein degradation. Of these, three HSP20 proteins were downregulated under the elevated CO2 condition, while the other 9 proteins were upregulated, such as DnaJ homolog subfamily A member 2 (DNAJA2), S-phase kinase-associated protein 1, and so on (Fig. 7B; see Table S6).

Basic carbon metabolism activities. We found 38 glycolysis-associated genes, of which 22 genes (15 upregulated, 7 downregulated) displayed differential expression...
under the elevated CO₂ condition. Interestingly, genes encoding rate-limiting enzymes of the glycolysis pathway, such as hexokinase (HK) and pyruvate kinase (PK), were significantly upregulated when S. marinoi was cultivated under the elevated CO₂ condition (Fig. 8; see Table S7). This result indicated that glycolysis seemed to be facilitated by transcriptional upregulation of HK and PK. Meanwhile, our proteomic analysis identified 30 glycolysis-associated proteins, among which the expression of 8 proteins changed significantly, but HK and PK proteins displayed no differential expression under the elevated CO₂ condition (Fig. 8; see Table S7). Therefore, from the proteomic level, we concluded that the glycolysis pathway did not change significantly under the elevated CO₂ condition.

Furthermore, we identified 65 TCA cycle-associated genes. Of these, 36 genes, such as citrate synthase (CS) and malate dehydrogenase (MDH), were upregulated under the elevated CO₂ condition, while 11 genes, annotated as pyruvate dehydrogenase E1 component alpha subunit (PDHA), dihydrolipoamide dehydrogenase (DLD), dihydrolipoamide acetyltransferase (DLAT), etc., were downregulated (Fig. 8; see Table S7), suggesting that the TCA cycle was enhanced under the elevated CO₂ condition. Similar results...
FIG 8 Heatmap showing changes in expression levels of genes/proteins involved in glycolysis, the pentose phosphate pathway, the TCA cycle, fatty acid metabolism, and N-glycan biosynthesis. The heatmap color scale represents homogenized gene or protein expression values (log2-fold change) on a column z-score, increasing from blue (lowest) to red (highest). NA, not available.
were also found in our proteomic database that the TCA cycle was accelerated. Expression profiling showed that 24 proteins involved in the TCA cycle (in a total of 47 proteins) displayed upregulation in response to ocean acidification, but 3 proteins showed opposite expression patterns (see Table S7).

In addition, we searched genes involved in the pentose phosphate pathway and found that the transcript expression of 9 genes (4 upregulated, 5 downregulated) were significantly changed under the elevated CO₂ condition (Fig. 8; see Table S7). Meanwhile, iTRAQ analysis indicated that 3 proteins annotated as 6-phosphogluconate dehydrogenase (PGD), transketolase (TKT), and transaldolase (TALA) displayed significantly differential expression under the elevated CO₂ condition (Fig. 8; see Table S7). Glucose-6-phosphate 1-dehydrogenase (G6PDH) has been generally considered the rate-limiting enzyme for the pentose phosphate pathway. In this study, we detected that the G6PDH gene was downregulated transcriptionally but no change was detected at the protein level under the elevated CO₂ condition (Fig. 8; see Table S7). Taken together, our data sets indicated no upregulation and possibly slight downregulation of the pentose phosphate pathway under the elevated CO₂ condition.

Fatty acid metabolism in eukaryotic microalgae mainly includes de novo synthesis of fatty acid in chloroplasts, elongation of fatty acids in mitochondria or the endoplasmic reticulum, biosynthesis of unsaturated fatty acids in chloroplasts or the endoplasmic reticulum, and oxidation of fatty acids in mitochondria. In this study, seven genes associated with de novo synthesis of fatty acid showed significantly higher expression under the elevated CO₂ condition. Of these, five genes encoding two acetyl coenzyme A (acetyl-CoA) carboxylases (ACCase), malonyl CoA-(acyl-carrier protein) transacylase (MCAT), 3-oxoacyl-(acyl carrier protein) synthase III (KASIII), and 3-oxoacyl-[acyl-carrier protein] reductase (KAR) were downregulated under the elevated CO₂ condition, while two genes encoding 3-oxoacyl-(acyl carrier protein) synthase II (KASII) were upregulated (Fig. 8; see Table S7). Proteomic analysis indicated that three proteins encoding MCAT, enoyl-(acyl carrier protein) reductase I (EARI), and KASII were differentially expressed between the control group and the experimental group. Among them, MCAT and EARI proteins exhibited downregulation expression profiles under the elevated CO₂ condition, while KASII protein showed the opposite expression pattern (Fig. 8; see Table S7). We also searched genes involved in the biosynthesis of unsaturated fatty acid and found that stearoyl-CoA desaturase (SCD) and omega-6 fatty acid desaturase (FAD2) showed strong upregulation under the elevated CO₂ condition. However, in our proteomic database, we only detected acyl-(acyl carrier protein) desaturase (ADD) protein, which displayed no significant differential expression under the elevated CO₂ condition (Fig. 8; see Table S7).

For fatty acid elongation, we found 30 related genes from our transcriptomes, among which 19 genes displayed upregulation whereas three downregulation (Fig. 8; see Table S7). At protein level, 6 of 17 related proteins detected were strongly upregulated under the elevated CO₂ condition, such as long-chain acyl-CoA synthetase (ACSL) (Fig. 8; see Table S7). For fatty acid oxidation, we obtained 16 associated genes. Among these, 9 genes were upregulated under the elevated CO₂ condition, while the other 4 genes were downregulated (Fig. 8; see Table S7). Totally, 13 proteins related to fatty acid oxidation were identified from our proteomic database. Among these proteins, six proteins showed significantly upregulation under the elevated CO₂ condition, while two proteins annotated as ACAT and acetyl-CoA acyltransferase 1 (ACAA1) exhibited opposite expression pattern (Fig. 8; see Table S7).

N-glycosylation is a major co- and posttranslational modification in the synthesis of proteins in eukaryotes. N-glycan processing occurs in the secretory pathway and is essential for glycoproteins destined to be secreted or integrated into the membranes (21). Here, we identified 42 genes associated with N-glycan biosynthesis, among which 32 genes (28, upregulation; 4, downregulation) showed significant regulation under the elevated CO₂ condition, such as dolichol-phosphate mannosyltransferase (DPM), dolichol kinase (DOLK), and so on (Fig. 8; see Table S7). iTRAQ analysis showed that 8 proteins (a total of 15 proteins) involved in N-glycan biosynthesis were differentially
expressed between the control group and experiment group. Of note, all these DEPs exhibited upregulation under the elevated CO\textsubscript{2} condition (see Table S7). These analyses showed that N-glycan biosynthesis was enhanced under the elevated CO\textsubscript{2} condition, at both the transcriptional and the translational levels.

**ROS homeostatic pathway, calcium signaling, and programmed cell death.** Reactive oxygen species (ROS) are typical by-products mainly due to the activation of NADPH oxidase, which is a respiratory-burst oxidase, catalyzing the NADPH-dependent reduction of O\textsubscript{2} into the superoxide anion (O\textsubscript{2}^\textsuperscript{-}) in cells (22). We searched for ROS homeostatic genes and found 26 were significantly regulated under the elevated CO\textsubscript{2} condition. Of these, 18 genes, were upregulated, while 8 genes were downregulated under the elevated CO\textsubscript{2} condition (Fig. 6; see Table S8). Moreover, we identified 15 DEPs associated with ROS homeostasis, and all of these showed upregulation under the elevated CO\textsubscript{2} condition (Fig. 6; see Table S8). Besides, we also found 18 genes and 4 proteins associated with calcium signaling. With the exception of those for calcium-transporting ATPase, all of these DEGs were upregulated (Fig. 6; see Table S8). Meanwhile, two proteins annotated as calcium-transporting ATPase and calcium-translocating P-type ATPase were found to be upregulated under the elevated CO\textsubscript{2} condition (Fig. 6; see Table S8).

Programmed cell death (PCD) is a form of autocatalyzed cell death mediated by expression of caspase-like and often leads to apoptosis (23). Comparative genome and EST analysis have characterized two distinct families of caspase-like proteins: paracaspase (PCA) and metacaspase (MCA) (24). Thus far, experiments to link PCD to caspase-like proteins have shown that only MCAs corresponded to autolysis in some species of marine diatoms (25, 26). In this study, three genes encoding MCA were found significantly downregulated under the elevated CO\textsubscript{2} condition, but all MCA proteins identified showed no changes in expression level under an elevated CO\textsubscript{2} condition (Fig. 6; see Table S8).

**DISCUSSION**

At present (i.e., in 2020), the content of CO\textsubscript{2} in the atmosphere exceeds 410 ppm, which is nearly 50% higher than that in the preindustrial period. These high growth rates are unprecedented in the past 55 million years of the geological record (27). About 25% CO\textsubscript{2} is absorbed by the ocean, where it reacts with seawater to form a weak acid environment, causing surface ocean pH to drop by ~0.004 U each year (1). The rate of this change is cause for serious concern, since many marine organisms may not be able to evolve quickly enough to adapt to these changes (28). There have been a few studies that examined the difference between short-term and long-term responses of diatoms communities to climate change variables. Tatters et al. (29) found that “artificial” communities comprising clonal isolates of diatoms conditioned to elevated-pCO\textsubscript{2} conditions for 1 year yielded the same general competitive responses observed in the short-term (2 weeks) experiment with the natural marine diatom community. In another long-term experiment with freshwater diatoms community, there was no evidence for evolutionary change after over 750 generations at an elevated pCO\textsubscript{2} (30). Similarly, 100 generations of selection by elevated pCO\textsubscript{2} resulted in little adaptation or clade selection in the diatom *Thalassiosira pseudonana* (31). These results suggest that evolutionary adaptation to elevated CO\textsubscript{2} after long-term exposure may be conserved in natural diatoms communities, without substantial evolutionary change. In the case of elevated pCO\textsubscript{2}, although some small phenotypic changes have been observed in the long-term incubations of the diatom species *Nitzschia lecointei* and *Phaeodactylum triquetrum*, since no evolutionary response has been detected, we cannot confirm whether it is a plastic or an adaptive response (32, 33). Therefore, it is important to study the effects of elevated pCO\textsubscript{2} on diatoms in both the short and the long term, as well as evolutionary response.

**Overall response under the elevated CO\textsubscript{2} condition.** In this study, 32,389 unigenes and 1,826 proteins were significantly regulated under the elevated CO\textsubscript{2} condition, which accounted for 48.5% of total genes and 25.9% of total proteins, respectively.
Table 1). The lower growth rates of S. marinoi and remarkable changes in gene and protein expression under the elevated CO2 condition found in this study together clearly indicated that the physiological and biochemical characteristics of S. marinoi cells were affected by elevated pCO2. Therefore, the seawater inorganic carbon system that approximates conditions expected for the end of this century probably has a negative and significant impact on the growth of S. marinoi. An earlier investigation focusing on a unialgal fouling diatom Navicula distans found that warming (caused by the increase atmospheric pCO2 at the end of this century) and acidification synergistically significantly inhibited its growth (34). In contrast, another survey using model diatom Thalassiosira pseudonana found that elevated pCO2 had a positive effect on its growth under subsaturating growth light (35). For S. costatum, a congenic to our study species, growth was found to be enhanced at an enriched pCO2 of 750 μatm (36) but not at 800 μatm (37). These conflicting results are clear evidence that all species are not the same in responding to elevated CO2, which has significant implications in impacting phytoplankton community structure and hence the biogeochemistry cycle of the entire marine ecosystem. Through elaborate transcriptomic and proteomic analyses, a wide range of metabolic impacts was found for elevated CO2 on S. marinoi, but the results overall indicated that elevated pCO2 and the consequent decreased pH caused negative effects on growth and various physiological processes.

Consistent with the findings in previous investigations, our study showed that transcriptome analyses yield more data than iTRAQ quantification proteomics analyses. Furthermore, gene regulation can occur at transcriptional or translational or posttranslational level and, as such, transcriptomic profiles may not track proteomic profiles (38, 39). In the present study, the majority of proteins detected (60.8%, a total of 6,831 proteins) were correlated with transcripts (Table 1 and Fig. 2), whereas the other nearly 40% were not correlated, and more transcripts had no protein support. Therefore, the transcriptomic data provided a more comprehensive insight into cell metabolism, while the coherent part of the proteomic data provides verification. Combining proteomic and transcriptomic data sets allows us to yield a comprehensive insight into cell physiology and data robustness in many cases.

Enhancement of transcription and translation activities under the elevated CO2 condition. Three RNA polymerase (Pol) complexes—Pol I, II, and III—transcribe the genetic information (40). Pol I, II, and III are essential for the synthesis of ribosomal RNAs (rRNAs; ~75%), messenger RNAs (mRNAs; 5 to 10%), and short structured RNAs (mainly 5S rRNA [5S rRNA] and transfer RNAs [tRNAs], ~15%), respectively (41). In S. marinoi, both our transcriptome and proteome showed that Pol I, II, and III transcript or protein abundances were increased under the elevated CO2 condition (Fig. 9), indicating that RNA synthesis was promoted. Interestingly, the increased RNA synthesis seems to be offset by the increased degradation potential, as suggested by the upregulation of the RNA degradation pathway genes. This might be due to the need to accelerate reshuffling of the cellular metabolic pathways under the elevated CO2 conditions. In support of this possibility are the remarkable dynamics of different metabolic pathways revealed by the “omic” data sets.

One of the metabolic pathways strongly influenced by OA was protein synthesis. In eukaryotic cells, the initiation of protein synthesis can be subdivided into three steps: first, assembling the 40S ribosomal subunit with an initiator methionyl-tRNA (Met-tRNA); second, binding the resulting large macromolecular complex to the start codon of mRNA; and third, the 60S ribosomal subunit participating in generating a translation-competent ribosome (42). The previous publication has proved that all these three steps are facilitated by eukaryotic translation initiation factors (eIFs) (43). Our data showed that most genes or proteins associated with eIFs in S. marinoi were upregulated under the elevated CO2 condition (Fig. 7B), indicating increased translation initiation activity and hence increased protein synthesis. Translation initiation of most mRNAs in eukaryotic cells is accomplished by ribosome. After this, newly synthesized polypeptides are transported across or integrated into the ER membrane for protein folding, secretion, and glycosylation and facilitate the degradation of misfolded
proteins in the ER lumen (44, 45). There is evidence that Sec61/SecY complex, which surrounds the polypeptide chain during its passage across the ER membrane, is the essential element for protein translocation (46, 47). Our transcriptomic and proteomic data showed that Sec61 complex was upregulated under the elevated CO2 condition (Fig. 9). Furthermore, in both RNA-seq and iTRAQ analyses, we found that protein processing in the ER was also strongly accelerated under the elevated CO2 condition (Fig. 9).

In eukaryotes, more than 50% of proteins are glycoproteins (48). The N-glycan biosynthesis that takes place in the ER is tightly implicated in the folding and retention, stability and turnover, intracellular trafficking, recognition and degradation, and physiological function of glycoproteins (49). In addition, N-glycan can also act as recognition tags to allow glycoproteins to interact with glycosyltransferases, glycosidases, and lectins (50). Interestingly, under elevated pCO2, both transcriptomic and proteomic analyses indicated that N-glycan biosynthesis was upregulated, indicating elevation of N-glycan formation (Fig. 9), which may promote the modification of glycoproteins. Together, these results suggest that elevated CO2 induced an increase in protein synthesis and processing. ER-associated degradation (ERAD) pathway is crucial for maintaining proteostasis by identifying and degrading normal and misfolded proteins (51–53). Our results suggest the molecular machinery for ERAD was activated under the elevated CO2 condition. This is consistent with our postulation of the need to accelerate metabolic reconfiguration and in addition the need to maintain proteostasis in the
cells. More incorrect folding or assembly of proteins might happen under elevated CO₂, imposing the need to degrade these misfolded proteins in the ERAD system and simultaneously synthesize new proteins.

The elevated protein synthesis and degradation, although likely a useful strategy for the cells to cope with elevated CO₂, would divert cellular resources from other cell functions such as proliferation. In Escherichia coli, studies have suggested that increased protein production leads to reduced cell growth and stalled cell cycle (54, 55). This resource reallocation, or trade-off between stress coping and proliferation, seems to occur in S. marinoi. After acclimation for 10 generations, Thangaraj and Sun (56) found that OA did cause activated protein processing machinery because of increased amino acid synthesis and nitrogen assimilation in Skeletonema dohrnii. This result is consistent with our study, so we speculate that activated protein processing machinery may be an adaptation of Skeletonema species to OA. Population growth of S. marinoi declined under the elevated CO₂ condition, while the capacity of protein production was expanded. Whether a similar regulatory mechanism exists in other diatoms warrants further investigation.

**Downregulation of CCM and photosynthesis.** Photosynthetic carbon fixation depends on ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), the enzyme that catalyzes the first and critical step of both photosynthetic CO₂ assimilation (57). Because Rubisco is very inefficient, CO₂ concentration in the natural seawater (ca. 10 to 15 μM) is inadequate (half-saturation concentration, >25 μM) (58). Usually, diatoms and many other lineages of phytoplankton use carbon-concentrating mechanism (CCM) to acquire carbon against the environment-cell uphill gradient. This includes C₄ photosynthesis, carbonic anhydrase (CA), and bicarbonate transporters. C₄ pathway has been proven to exist and operate in diatoms (12, 59, 60). Since CCM requires energy, it has been a topic of debate whether OA would reduce CCM capacity and hence save energy (10, 61). In this study, we found that S. marinoi indeed possesses C₄ pathway and that it was indeed downregulated under the elevated CO₂ condition. The C₄ pathway involves phosphoenolpyruvate carboxylase (PEPC) which fixes bicarbonate into the oxaloacetate (OAA, C₄ acids) in the cytoplasm for subsequent transport into the chloroplast (62). In general, diatoms have two PEPC proteins, which may have different functions due to different subcellular localization (63). Consistent with previous studies, our study revealed the presence of two genes encoding PEPC (named PEPC1 and PEPC2) in S. marinoi. Analysis using CELLO (64) predicts that PEPC1 protein occurs in the cytoplasm, whereas PEPC2 protein occurs in mitochondria. Therefore, it is likely PEPC1, not PEPC2, that functions in the C₄ pathway associated with photosynthetic carbon fixation. The downturn of CCM is also evident in the decreased protein expression of CA. However, if there was energy saved from lowered CCM machinery, it was not reinvested in photosynthesis, because the large subunit of Rubisco (RBCL) also displayed weak downregulation (albeit not significant) (Fig. 9). Combining proteomics and transcriptomic data has yielded the insight that CCMs is probably weakly downregulated by the elevated pCO₂, and the weak downregulation of RBCL indicated a slight decrease in carbon fixation capacity.

Consistent with the above assessment, both proteomic and transcriptomic analysis showed that oxygen-evolving complex (OEC), light-harvesting complex I chlorophyll a/b binding proteins (Lhca), cytochrome c₆ (Cytc6), and ferredoxin (Fd) were downregulated under elevated pCO₂ (Fig. 9), indicating reduction of linear electron flow (LEF) and cyclic electron flow (CEF), resulting in the decrease of ATP synthesis. In accordance with this, elevated pCO₂ caused the downregulation of chloroplastic ATP synthase.

**Accelerated energy metabolism.** Energy metabolism via glycolysis, the TCA cycle, the pentose phosphate pathway (PPP), mitochondrial electron transport, and oxidative phosphorylation through which ATP, NAD(P)H, and various intermediate carbon compounds are generated is necessary for cell maintenance and growth (65). Increased acidity of seawater associated with elevated pCO₂ can disturb intracellular pH stability, so that diatom may have a greater need for energy in order to maintain cellular homeostasis (5). A previous report, focusing on diatoms and coccolithophores, indicated that
elevated pCO₂ could increase the activity of glycolysis, the TCA cycle, and β-oxidation of the fatty acid pathway (66). Transcriptomic analysis of 2% against 0.4% CO₂-cultured Coccomyxa subellipsoidea C-169 has unveiled that the TCA cycle, glycolysis, and oxidative phosphorylation were predominantly upregulated under increased pCO₂, and this pattern could provide more energy and intermediate carbon compounds to respond to environmental stress (67). In this study, most DEGs or DEPs identified in the mitochondrial respiration were upregulated under the elevated CO₂ condition, indicative of enhanced energy metabolic capacity.

Lipid synthesis and consumption are also important energy pathway. The effect of CO₂ variation on fatty acid content and composition has been previously reported for Skeletonema, but there seem to be differences among different species. For instance, in S. pseudocostatum, the total fatty acid concentration remarkably decreased under elevated pCO₂ (9). In contrast, high temperature and pCO₂ (25°C, 1,000 ppm) increased the productivity of fatty acid in S. dohrnii (11). In our iTRAQ study, we found that fatty acid metabolism was significantly enriched under the elevated CO₂ condition (Fig. 4). Most DEGs/DEPs identified in de novo fatty acid biosynthesis were downregulated under elevated CO₂, a finding indicative of reduced fatty acid synthesis capacity (Fig. 8 and 9).

However, synthesis of long-chain fatty acids (LCFAs) and polyunsaturated fatty acids (PUFAs) seems to be promoted by elevated CO₂ in our study. It has been characterized that KASII mediates the elongation of C₁₆₆-ACP to C₁₈₀-ACP through the condensation of two-carbon units in the de novo fatty acid biosynthesis pathway (68). Subsequently, the C₁₈ acyl may serve as precursors for the elongation of LCFAs. Interestingly, both the KASII transcript and protein were upregulated, as were genes/proteins associated with LCFAs, such as ACSL, ELOVL5, VLKAR, and VLECR (Fig. 8), which could potentially promote LCFA production. Besides, from the transcriptomic, we found that 2 genes encoding stearoyl-CoA desaturase (SCD) and omega-6 fatty acid desaturase (FAD2) were upregulated, which, if verified to be so at proteins and activity levels, indicates promotion of PUFA production. Consistent with the finding in the present study, Liang et al. (16) document that elevated pCO₂ favored synthesis of PUFAs in Dunaliella salina. To use storage lipids efficiently, fatty acids need to be completely converted from acyl-CoA to acetyl-CoA by fatty acid β-oxidation (69). Interestingly, many genes/proteins associated with β-oxidation were upregulated, which could potentially generate more energy for the survival and growth of S. marinoi cells. As previously reported, β-oxidation is a potential site for ROS formation, because the intermediates and by-products of β-oxidation can interfere with antioxidant mechanisms (70). Therefore, we speculated that β-oxidation, such as complexes I, II, III, and IV, could also promote ROS production. Taken together, the reduction in total fatty acids and increases in LCFAs, PUFAs, and β-oxidation under acidification may affect cell growth and survival, the turnover of membrane lipids, and the rapid mobilization of storage lipids.

Nitrogen uptake and metabolism. In our study, nitrate was the only N source of S. marinoi. From the transcriptomic data set, we found that nitrate transporter (NRT), nitrate reductase (NR), and nitrite reductase-ferredoxin (NiR) were all upregulated under the elevated CO₂ condition, but there was no significant change in protein levels (Fig. 5; see Table S3), raising uncertainty about increasing N uptake. However, carbamoyl phosphate synthase (CPS) and glutamine synthetase (GS) were all upregulated under elevated CO₂ in both transcriptional and translational levels (Fig. 5; see Table S3), a finding indicative of the acceleration of N assimilation and urea cycle.

It is interesting to note that the inorganic phosphate transporter protein was also upregulated under elevated pCO₂. This is consistent with the intensification of energy metabolism discussed earlier, which may require more phosphorus. We also found that the ABC transporter families displayed significant regulation. The ABC transporters bind ATP and use energy to shuttle a great variety of molecules across different cellular membranes (71). Metals are indispensable for microalga cells to perform their cellular functions, since they not only serve as components for photosynthetic system and constituents of vitamins but also act as cofactors for enzymes involving in CO₂ fixation, nitrate reduction, phosphorus acquisition, and DNA transcription (72). Combining our
transcriptomic and proteomic data, we found that metal uptake, such as calcium, potassium, zinc, and iron, showed significantly upregulation under the elevated CO$_2$ condition. This suggests that _S. marinoi_ might require more metal ions to cope with decreased pH. Alternatively, the upregulation of the uptake genes might indicate compromised bioavailability of metals under acidification, as demonstrated in the case of iron (73).

**Cross talk among ROS, calcium signaling, and programmed cell death.** In plants, ROS-coupled calcium signaling is largely recognized to be important signal mediators, which is involved in many cellular processes, including growth, development, differentiation, programmed cell death (PCD), and so on (74–76). In this study, we particularly focused on the cross-relationship among ROS, calcium signaling, and PCD. An abundance of publications indicates that Ca$^{2+}$ or calmodulin causes activation of NADPH oxidase and, in consequence, augmentation of ROS production (77–79). Previous studies have shown that diatoms use a novel and highly sensitive Ca$^{2+}$-dependent signaling pathway to sense and respond to abiotic environmental signals (80, 81). For example, aequorin-transformed _P. tricornutum_ cells exhibit rapid cytosolic Ca$^{2+}$ elevations in response to mechanical stimuli (81). After a period of phosphorus (P)-limited growth, phosphate resupply results in rapid, transient elevations in the cytosolic Ca$^{2+}$ of _P. tricornutum_ (80). In our _S. marinoi_ transcriptomic study, we found that the calcium-transporting P-type ATPase and Ca$^{2+}$-permeable channels were all upregulated under the elevated CO$_2$ condition (Fig. 6; see Table S8), which could potentially promote a rapid influx of Ca$^{2+}$ into the cytosol. Interestingly, some Ca$^{2+}$ efflux genes, such as two-pore calcium channel protein 1 and calcium-transporting ATPase, also exhibited upregulation under the elevated CO$_2$ condition. The potential increase of Ca$^{2+}$ traffic might indicate accelerated calcium signaling under the elevated CO$_2$ condition. The previous study also showed that OA can affect environmental sensing of microalgae and thus enhance cellular Ca$^{2+}$ fluxes (82). Therefore, we speculate that the activated Ca$^{2+}$ transport and calcium signaling may be responses to elevated pCO$_2$.

ROS can react very rapidly with proteins, nucleic acids, and lipids, thereby resulting in severe cell damage and cell death (83). Potentially toxic free radicals can be removed by ROS scavenging systems composing of diverse enzymatic antioxidants such as glutathione peroxidase (GPX) and some small molecules of nonenzymatic antioxidants such as ascorbic acid (84). That is, scavenging mechanisms hold the cellular steady-state level of ROS under tight control. RNA-seq analysis suggested that the upregulation of NADPH oxidase triggered the augmentation of ROS production. Correspondingly, under the elevated pCO$_2$, most DEGs and all DEPs annotated as enzymatic antioxidants were upregulated, indicating enhanced ROS scavenging capacity. It has been proposed that PCD can be triggered by higher doses of ROS (85, 86). In marine diatom, metacaspase (MCA) is considered the most common molecular marker for PCD (25, 26). However, while MCA genes showed significant downregulation under the elevated CO$_2$ condition, MCA proteins displayed no significant change in expression. Despite the discrepancy, both transcriptomic and proteomic data sets agreed that elevated pCO$_2$ did not induce PCD. This might have been made possible because _S. marinoi_ was able to maintain stable ROS levels due to its upregulated ROS scavenging capacity.

**Conclusions.** Our study showed that elevated CO$_2$ repress photosynthesis and growth of _S. marinoi_, and the “omic” data suggested that this might be due to compromised photosynthesis and fatty acid biosynthesis in the chloroplast and raised mitochondrial energy metabolism. Meanwhile, our data revealed the upregulation of nitrogen metabolism, transcriptional activity, and translational activity, implying a heightened potential of protein synthesis. The increased protein synthesis and energy metabolism might be required for cells to maintain intracellular homeostasis in face of decreasing pH. In addition, elevated pCO$_2$ seemed to promote ROS production and anti-oxidative stress capacity in _S. marinoi_, and apparently due to balance of ROS and antioxidant, no PCD induction was observed. Overall, elevated pCO$_2$ seems to cause massive gene expression reconfiguration to increase investment in protein synthesis, energy metabolism, and antioxidative
stress defense, likely to maintain pH homeostasis and population survival. Although the evolutionary adaptation of diatoms to OA after long-term exposure may be conserved, it is noteworthy that some phenotypic changes were observed between short-term and long-term incubations. Therefore, further research should be focused on long-term physiological responses of *S. marinoi* to OA.

**MATERIALS AND METHODS**

**Algal culture and CO₂ manipulation.** The strain of *S. marinoi* was obtained from the Algae Culture Center of Key Laboratory of the Marine Environment and Ecology, Ministry of Education, Ocean University of China. Batch cultures of *S. marinoi* were maintained in 1-liter sterilized Erlenmeyer flasks with 500 ml of f/2 medium using the 0.22-μm pore-size Millipore filtered and autoclaved natural seawater (from Jiaozhou Bay, a shallow semiclosed bay near Qingdao city on the Yellow Sea coast in the north of China) at 20°C under a 12-h/12-h light and dark cycle, with a light intensity of ~80 μmol of photons m⁻² s⁻¹.

To set up CO₂ treatment, *S. marinoi* cells in the midexponential phase were collected by centrifugation (4,000 × g at 20°C for 10 min), and the cell pellet was suspended and inoculated into six Erlenmeyer flasks with an initial cell density of 1 × 10⁶ cells ml⁻¹. Prior to inoculation, the cultures were aerated for 24 h at two different CO₂ levels (ca. 400 and 1,000 ppm) at an aeration rate of 300 ml min⁻¹, corresponding to the current approximate CO₂ level in the atmosphere and the predicted level in 2,100, respectively. The outdoor air was used for the ambient CO₂ treatment (control), whereas 1,000-ppm CO₂ treatment (with <5% variation) was achieved by mixing 99.99% CO₂ with zero-CO₂ air using plant CO₂ chambers (HP400G-D; Ruihu Instrument & Equipment, Ltd., Wuhan, China). The two different CO₂ mixtures were pumped into the culture flasks through 0.22-μm Milllex-GP sterile syringe filters (SLGP033RS; MilliporeSigma, Burlington, VT) using air compression pumps. Each treatment was made in triplicate. The cell density was determined daily by using a phytoplankton counting chamber (Sangon, Shanghai, China). pH values were monitored daily at the same time as sampling using an Orion VersaStar Pro multiparameter benchtop meter (Thermo Fisher Scientific, Inc., Waltham, MA) performed with three points of pH calibration. The following formula was used to calculate intrinsic growth rates: μ = ln(N/N₀)/(tₙ/t₁), where N₁ and Nₙ are the cell densities at times t₁ and tₙ, respectively (87).

**Sample collection, RNA extraction, and RNA-seq.** When the cultures entered the late exponential phase (day 5), *S. marinoi* cells were harvested by centrifugation at 5,000 × g for 10 min at 4°C for RNA and protein extraction. The samples were immediately frozen in liquid N₂ and then stored at −80°C until subsequent extraction. Total RNA was extracted using 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The concentration of extracted RNA was assessed on NanoDrop (ND-2000 spectrophotometer; Thermo Scientific, Wilmington, DE), while the integrity was measured on an Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA).

Samples with an RNA integrity number of ≥7.8 were used for transcriptomic sequencing. Briefly, 1 μg of total RNA from each sample was used for enriching poly(A) mRNA to construct a paired-end RNA-seq library and subsequently processed for transcriptomic sequencing. Paired-end sequencing of 2 × 100-bp was performed on the BGISEQ-500 platform (BGI Genomics Co., Ltd., Wuhan, China) to obtain >50 million read pairs per sample. To obtain the clean data, low-quality reads, reads containing adapters, and reads with ambiguous “N” nucleotides were identified and removed. After the quality control procedure, the filtered reads were assembled de novo using Trinity software (v2.0.6) (88).

**Gene functional annotation and DEG analysis.** For functional annotation, all unigenes were mapped to seven public databases with a threshold E value of 10⁻⁰⁷. These databases included NCBI nonredundant nucleotide (Nr), NCBI nonredundant protein (Nr), Swiss-Prot, Pfam, Eukaryotic Ortholog Groups (KOG), Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG). For DEG analysis, RSEM (v1.2.8) with default parameters was used to estimate gene expression for each sample (89). The fold change values were calculated by the DESeq (an R package) (90), and the P values were adjusted for multiple testing (91) and statistical significance analysis (92). Genes with fold changes of ≥2 and adjusted P values (Q values) of ≤0.001 were selected as DEGs for subsequent analysis. GO annotation or KEGG functional enrichment analysis was performed using the phyper function of the R package to identify significantly enriched GO terms or KEGG pathway (false discovery rate [FDR] values of ≤0.01) associated with DEGs.

**Protein extraction.** Before protein extraction, frozen samples of *S. marinoi* were washed with phosphate-buffered saline (PBS) three times. Each pellet was subsequently homogenized in lysis buffer (8 M urea, 40 mM Tris-HCl [pH 8.0], 1 mM protease inhibitor, and 2 mM EDTA). After being placed on ice for 5 min, the lysate with 10 mM dithiothreitol was sonicated at 4°C for 2 min and centrifuged at 25,000 × g for 15 min at 4°C; the supernatant was then collected. The supernatant was mixed with a 4× volume of acetone. The mixture was vortexed, precipitated at −20°C overnight, and centrifuged at 25,000 × g for 15 min at 4°C to remove the supernatants. Precipitated proteins were dried at 4°C, redissolved in lysis buffer, sonicated (50 Hz) at 4°C for 2 min, and centrifuged at 25,000 × g for 15 min at 4°C. We then collected the supernatants and incubated the protein solutions at 56°C for 1 h to reduce the disulfide bonds. After cooling to room temperature, the protein solution was incubated with a final concentration of 55 mM iodoacetamide for 45 min in the dark for alkylation. Cooling acetone was added again, and the samples were precipitated at −20°C overnight and centrifuged to obtain the precipitate. After being
air dried at 4°C, the lysis buffer was used to solubilize protein pellets. The protein concentration and integrity were determined by a Bradford assay and SDS-PAGE.

**Protein digestion, iTRAQ labeling, and peptide fractionation.** The protein solutions (100 μg) were diluted four times with 100 mM trietahlylammonium bromide (TEAB). According to the manufacturer’s protocol, the protein was digested by Trypsin Gold (Promega, Madison, WI) with a protein/trypsin ratio of 40:1 at 37°C overnight. After trypsin digestion, the peptides were desalted and purified by using a Strata X C18 column (Phenomenex, Tianjin, China) and vacuum-dried. The peptides (100 μg) were dissolved in 30 μl of TEAB (0.5 M) with vortexing. After the iTRAQ reagents were thawed to room temperature, 50 μl of isopropyl alcohol was added to each reagent vial. The six peptide samples were labeled with 6 of 8 iTRAQ 8-Plex Reagents (reagents 113, 116, 117, 118, 119, an 121) and then fractionated on a high-pH reversed-phase high-performance liquid chromatography (RP-HPLC) pump system (Shimadzu LC-20AB; Shimadzu, Kyoto, Japan) coupled with Gemini C18 (4.6 × 250 mm; particle size, 5 μm) columns (Phenomenex, Tianjin, China).

**LC-MS/MS analysis.** Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed on an UltiMate 3000 UHPLC system (Thermo Fisher Scientific, San Jose, CA) coupled to a Q-Exactive HF-X mass spectrometer (Thermo Fisher Scientific) in data-dependent acquisition (DDA) mode by nano-electrospray ionization. Briefly, peptides in buffer A (2% acetonitrile, 1% formic acid) were injected to a homemade nanocapillary C18 column (75 μm × 25 cm; particle size, 3 μm) with a 50-min linear gradient from 5 to 80% buffer B (98% acetonitrile, 0.1% formic acid) at a flow rate of 300 nl min⁻¹. The gradient began at 5% buffer B; increased to 25% in 40 min, 25 to 35% in 5 min, and 35 to 80% in 2 min; was maintained at 80% for 2 min; and finally returned to 5% in 1 min and equilibrated for 6 min.

For MS analysis, the Q-Exactive HF-X mass spectrometry was operated in DDA mode with an electrospray voltage of 2.0 kV. MS data were acquired in full scan mode (350 to 1500 m/z) with a resolution of 60,000 and an automatic gain control (AGC) threshold of 3 × 10⁶. From the full MS scan, the 20 most abundant precursor ions above a threshold ion count of 10,000 were selected as MS/MS data for higher-energy collisional dissociation fragmentation at a resolution of 15,000 (at 100 m/z), a normalized collision energy of 30%, a dynamic exclusion time of 30 s, and an AGC target value of 1 × 10⁵.

**Protein identification and bioinformatics analysis.** For protein identification, the raw MS/MS data were submitted to a Mascot (v2.3.02) search against the aforementioned transcriptome database of *S. marinoi*. The final selected protein must contain at least one unique peptide. The iQuant software was used for protein quantification (93). The iQuant settings were set according to the “picked protein FDR strategy” with an FDR value of 0.01 (94). A protein with a fold change of >1.2 and a P value of <0.05 was accepted as the differentially expressed proteins (DEPs). To deeply analyze the DEPs and explore the potential mechanism of ocean acidification on *S. marinoi*, DEPs were used to perform GO annotation and KEGG pathway enrichment.

**Statistical analysis.** To evaluate the statistical significance of the differences observed between the ambient CO₂ group and the elevated CO₂ group, variance analysis was carried out by using SPSS 17.0 with one-way ANOVA, followed by Tukey’s post hoc tests. A probability (P) value of <0.05 was considered the threshold of statistical significance.

**Data availability.** The transcriptomic sequencing data in this study have been submitted to GenBank’s Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/) under accession number PRJNA661548. The mass spectrometry-based proteomics data have been deposited in the ProteomeXchange consortium via PRIDE under the data set identifier PXD021416.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, XLSX file, 0.2 MB.

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M.Z. and T.M. designed and performed experiments. M.Z., Y.Z., and S.L. wrote the manuscript.

We declare that there are no conflicts of interests regarding this article.

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